EXHIBIT B

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Regulation of Gene Expression *in Vivo* by Liposome-mediated Delivery of a Purified Transcription Factor*

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Robert J. Debs‡§, Leonard P. Freedman¶‖, Sarah Edmunds‡, Karin L. Gaensler‡, Nejat Düzgüneş‡**, and Keith R. Yamamoto¶

*From the ‡Cancer Research Institute and the Departments of **Pharmaceutical Chemistry and ¶Biochemistry and Biophysics, University of California, San Francisco, California 94143-0128*

We describe a procedure for assessing the functional activity *in vivo* of a glucocorticoid receptor derivative, T7X556, a mammalian transcriptional regulator that has been overexpressed in *Escherichia coli* and purified to homogeneity. The protein was assessed with DOTMA (*N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethyl-ammonium chloride) liposomes, which are internalized by cultured mammalian cells. T7X556 protein delivered in this manner localized rapidly to the nucleus and selectively enhanced expression from glucocorticoid response element-linked promoters, properties that are characteristic of this receptor derivative when it is synthesized endogenously in mammalian cells. Thus, *in vivo* activities of T7X556 were not disrupted by expression in bacteria or by biochemical purification. In general, liposome-mediated delivery may permit functional analyses of proteins that have been expressed in heterologous cells and manipulated *in vitro*.

DNA cloning and transfection methodologies have facilitated high level expression of proteins that are normally produced only in very low amounts; for example, mammalian transcription factors may be overexpressed in bacteria and purified for functional and structural characterization *in vitro* (1). A potential problem with this approach is that either the expression in heterologous cells or the manipulation of the protein during purification could lead to alteration, reduction, or loss of activities that are crucial to the action of the protein *in vivo*. Indeed, it is apparent that eukaryotic transcription factors undergo multiple complex interactions with other proteins or ligands *in vivo* (2, 3), suggesting that disruption of one or more crucial potential interactive surfaces might occur rather readily. Moreover, eukaryotic phosphoproteins or glycoproteins are commonly unmodified when synthesized in bacteria. Consistent with these concerns, *in vitro* assays of transcription and its regulation generally display weak activities relative to *in vivo* function. However, it has not routinely been determined whether such low activities reflect irreversible alterations in factor activities. In principle, this notion could be tested by introducing the purified factor into cells where it is normally synthesized and assessing the extent to which its potential activities have been retained. Unfortunately, techniques for delivery of protiens into cells have not been developed as fully as methods for delivery of nucleic acids.

The rat glucocorticoid receptor serves as an interesting example of a eukaryotic protein with multiple complex activities. Intrinsic to its functions as a signal transducer and transcriptional regulator (4–6) in mammalian cells, domains have been identified within the receptor that are essential for hormone binding, DNA binding, nuclear localization, transcriptional enhancement and repression, and for a novel property termed "protein inactivation" (7–12). After purification, some of these activities can be mimicked *in vitro*, usually at relatively low specific activities, whereas others have not yet been detected *in vitro*. To determine the extent to which bacterially expressed and purified derivatives of the glucocorticoid receptor retain *in vivo* activities, we have measured *in vivo* the transcriptional enhancement activity of a discrete segment of the receptor that has been expressed in bacteria and purified to homogeneity. We describe here a procedure for liposome-mediated delivery of the protein and protein-DNA complexes into cultured animal cells.

MATERIALS AND METHODS

Cell Culture—CV-1, an African green monkey kidney cell line, HTC, a rat hepatoma cell line, Ltk⁻, a murine fibroblast cell line, and primary rat embryo fibroblast cells were propagated in Dulbecco's modified Eagle's (DME)[1] medium supplemented with 10% fetal calf serum (FCS) in 7% CO_2 at 37 °C.

Plasmid Preparation and Characterization—Reporter plasmids (see Fig. 1*B*) have been described (13, 14); each expresses a chloramphenicol acetyltransferase (CAT) transcription unit, with 3′ end formation specified by the SV40 polyadenylation site. Expression is driven either by the mouse mammary tumor virus (MMTV) promoter (GMCO and G'MCO) or by the herpes simplex virus thymidine kinase promoter (GTCO and OTCO). GMCO contains the entire MMTV GRE, which encompasses five receptor footprint sites (15) and displays strong GRE activity; G'MCO is truncated to position-105 relative to the transcription start site, contains only one receptor footprint (15), and confers a weak GRE effect[2]; GTCO contains a 46-bp synthetic GRE (14) fused to position −109 of the thymidine kinase promoter.

Liposome Preparation—Liposomes were prepared as follows. 10 μmol of phosphatidylethanolamine (PE) dissolved in chloroform and 10 μmol of DOTMA (provided by Syntex Corp., Palo Alto, CA) dissolved in ethanol (for DOTMA·PE) or 20 μmol of DOMTA alone (for DOTMA) were evaporated to dryness on a rotary evaporator; 1 ml of buffer (50 mM Tris, pH 7.4, 0.5 mM EDTA, 50 mM NaCl, 100 μM $ZnCl_2$) was added, and the mixture was sonicated under argon in a bath sonicator (Laboratory Supply Co., Hicksville, NY) for 20 min.

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§ To whom correspondence should be addressed.

‖ Current address: Program in Cell Biology and Genetics, Memorial Sloan-Kettering Cancer Center, 1275 York Ave., New York, NY 10021.

[1] The abbreviations used are: DME, Dulbecco's modified Eagle's; FCS, fetal calf serum; CAT, chloramphenicol acetyltransferase; MMTV, mouse mammary tumor virus; GRE, glucocorticoid response element; PE, phosphatidylethanolamine; DOTMA, *N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride.

[2] D. De Franco and K. R. Yamamoto, unpublished data.

Both pure DOTMA and DOTMA·PE liposomes had mean diameters of 100 ±25 nm, as determined by laser light scattering in a Coulter N4 MD submicron particle analyzer.

Liposome-mediated Delivery and CAT Assays—CV-1, HTC, Ltk⁻, and rat embryo fibroblast cells were each plated at 10^6/100-mm dish; 20 h later, cultures were treated for 1 h with 100 μM chloroquine, washed twice, and 4 ml of culture medium was supplemented with 0.05% gentamycin and 100 μM chloroquine was added. Protein-DNA-liposome complexes were prepared by gently mixing the components and then suspending aliquots containing 25 nmol of DOTMA in 1 ml of serum-free DME medium; each dish of 10^6 cells received 1 ml of suspension, followed immediately by 4 ml of DME medium. After 5 h at 37 °C, 10 ml of DME medium supplemented with 10% FCS was added, and the cultures were incubated an additional 12 h, washed twice, and then incubated in fresh DME medium plus FCS for an additional 31 h. Finally, the cells were washed twice, scraped from the plate with a rubber policeman, centrifuged at 1000 × *g* for 5 min, resuspended in 135 μl of 250 mM Tris, pH 7.5, freeze-thawed three times, heated at 65 °C for 10 min, and centrifuged at 12,500 × *g* for 10 min. Supernatant protein concentration was determined, and CAT activity was measured on 20 μg of supernatant protein (16); after thin layer chromatography and autoradiography, activity was quantitated by scintillation counting of the acetylated [^{14}C]chloramphenicol species.

For experiments in which T7X556 and GTCO were delivered separately: (*a*) delivery of GTCO, then T7X556; CV-1 cultures (10^6 cells) were incubated for 3 h with 0.25 μg of GTCO associated with 12.5 nmol of DOTMA liposomes (L-GTCO) in DME medium; DME medium plus FCS was then added, and the cells were either rinsed six times over the next hour with DME medium plus FCS, or they were first incubated 12 h prior to rinsing. 125 μg of T7X556 plus 20 nmol of DOTMA liposomes (L-T7X556) in DME medium were added for 3 h; DME medium with FCS was then added, and the cultures were incubated for 3 h, washed twice, and incubated for an additional 17–41 h; (*b*) delivery of T7X556, then GTCO; cultures were treated as in *a* except the order of addition of L-GTCO and L-T7X556 was reversed. Extract preparation and CAT assays were as described above.

Immunoblotting—Cell extracts were prepared as described above but without the 65°C step and were fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with BUGR2, a receptor-specific monoclonal antibody (17).

RESULTS AND DISCUSSION

The X566 segment of the rat glucocorticoid receptor encompasses amino acids 407–556 of the 795-amino acid protein (Fig. 1*A*), a region that includes the DNA binding domain, a transcriptional enhancement domain, and a nuclear localization signal (7–9). When X556 is expressed in animal cells, it associates with specific sequences, termed GREs, and enhances transcription from nearby promoters (9); X556 lacks the hormone binding region, and therefore functions in the absence of ligands (18). In *Escherichia coli*, the X556 segment has been expressed from a T7 promoter, yielding a product termed T7X556, which has been shown to contain two zinc fingers and to associate specifically with GRE sequences *in vitro* (1).

We purified T7X556 to >95% homogeneity by ammonium sulfate precipitation followed by two chromatographic fractionations (1). To test whether the purified protein had retained potential for *in vivo* function, we used DOTMA liposomes to introduce T7X556 into CV-1 cells together with reporter plasmid GTCO, which carries a thymidine kinase promoter-CAT fusion gene linked to a GRE (Fig. 1*B*); CV-1 cells lack endogenous glucocorticoid receptor. As seen in Fig. 2*A*, T7X556 stimulated CAT expression in a dose-dependent manner, conferring up to 70-fold induction. No CAT expression was observed when T7X556 and GTCO were added to the cells in the absence of DOTMA liposomes. When we used liposomes comprised of a 1:1 mixture of DOTMA and PE, which have been employed to deliver DNA (19) and RNA

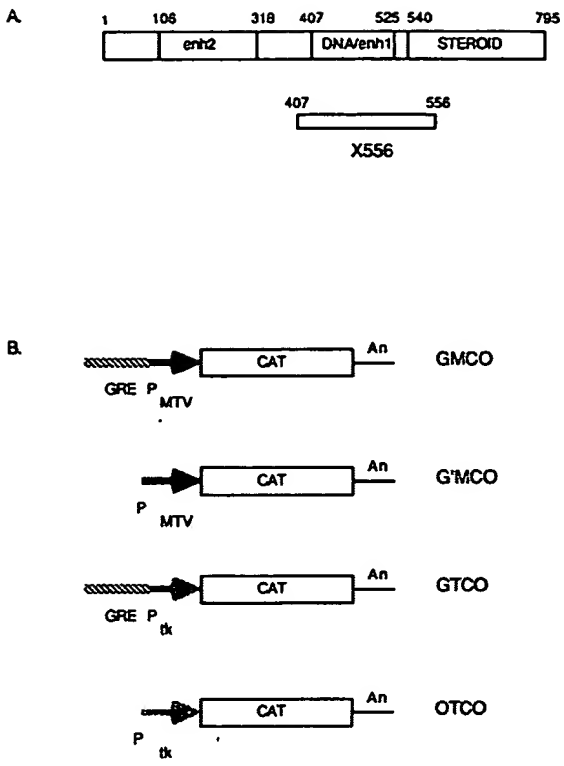

FIG. 1. **Receptor derivatives and reporter plasmids.** *A*, segments labeled in the upper diagram are sufficient for specific DNA binding, steroid binding, and transcriptional enhancement (*enh 1* and *enh 2*). The X556 fragment encompasses amino acids 407–556; when produced *E. coli* as T7X556, 10 and 14 nonreceptor amino acids are appended at the N and C termini, respectively. *B*, reporter plasmids (13, 14). CAT coding sequences, fused to the SV40 polyadenylation site (*An*), are driven by promoters either from MMTV (GMCO and G'MCO) or from thymidine kinase (*tk*) (GTCO and OTCO). GMCO and GTCO display strong GRE activity, G'MCO confers a weak GRE effect, and OTCO lacks GRE activity. See text for details.

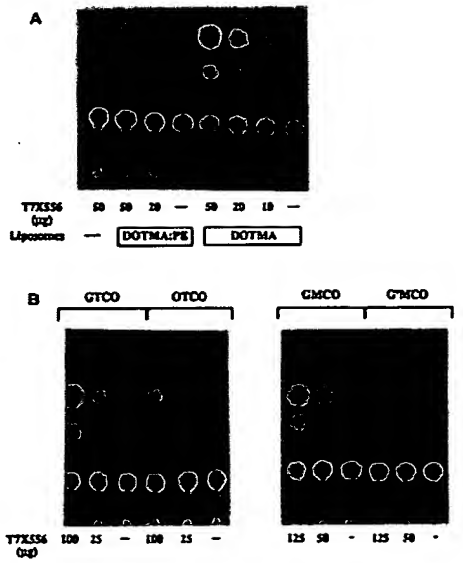

FIG. 2. ***In vivo* activity of purified T7X556 protein introduced into CV-1 cells.** *A*, CAT activities observed after the addition of 0.5 μg of GTCO together with various amounts of T7X556 in the absence or presence of DOTMA:PE or pure DOTMA liposomes. See text for details. *B*, CAT activities observed after pure DOTMA liposome-mediated delivery of 0.5 μg of GTCO or OTCO, or 2.5 μg of GMCO or G'CMO, together with various amounts of T7X556.

(20) into cells, only a low level of CAT expression was detected (Fig. 2*A*); we do not know why this mixture is so much less effective than pure DOTMA liposomes for delivering the protein. We also failed to observed T7X556 activity using non-DOTMA-containing liposome formulations (21) or other methods for introducing DNA into cell cultures including electroporation (22), scrape loading (23), protoplast fusion (24), DEAE-dextran (25), and calcium phosphate precipitation (25) (data not shown).

Enhancement of gene expression by liposome-delivered T7X556 closely mimics that conferred by X556 expressed in animal cells. For example, we assessed T7X556 activity on a series of CAT reporter plasmids (Fig. 1*B*) containing two different promoters (thymidine kinase or MMTV); transfection assays in cells expressing endogenous receptor (13, 26) show that reporter plasmid OTCO lacks a GRE, whereas G'MCO harbors a weak GRE, and GTCO and GMCO carry strong GREs. The relative levels of CAT expressed upon co-delivery of those plasmids together with T7X556 (Fig. 2*B*) indicate that the purified bacterial product confers relative activities that parallel those observed in cells synthesizing receptor endogenously. A slight increase in CAT activity was observed when OTCO was co-delivered with T7X556. This effect has not been investigated but may reflect either cryptic GREs on the plasmid vector or perhaps a level of DNA protection by nonspecifically bound protein, as T7X556 is present in great excess relative to OTCO in these experiments.

As shown in Fig. 2*A*, enhancement of receptor expression required high levels of T7X556 ($\geq$10 μg/10^6 cells). Studies with fluorescein-labeled (27) T7X556 (not shown) revealed that >50% of the protein became stably associated with the liposomes, indicating that protein-DOTMA lipsome complexes were forming efficiently. Approximately 20% of the cells appeared to take up the labeled protein. Moreover, the fluorescein-labeled protein accumulated strongly within CV-1 cell nuclei by 30 min after the addition of liposomes, as determined by fluorescence microscopy; such rapid kinetics of nuclear localization are similar to those reported for endogenously synthesized receptor derivatives (8). However, quantitative immunoblots of extracts of liposome-treated cells revealed that only about 0.2% of the added T7X556 was detected after 5 h; importantly, virtually all of the surviving protein was intact (Fig. 3). Based on these results, we calculate that exposure of 10^6 CV-1 cells to 10 μg of T7X556 on DOTMA liposomes results in association of approximately 3 $\times$ 10^5 molecules of T7X556/cell, which is about 10-fold above the endogenous glucocorticoid receptor content of normal cells. This is a maximum estimate, as it is unlikely that all of the detected protein is actually localized properly. Thus, while liposome delivery is inefficient, the T7X556 protein that is introduced into the cells appears to be intact and to display levels of activity similar to that of endogenously expressed protein.

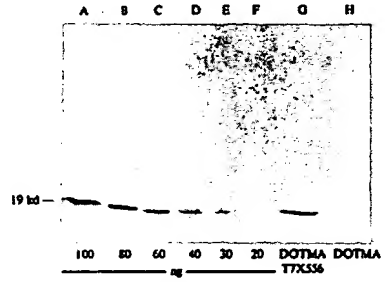

FIG. 3. **Quantitation of T7X556 delivered into CV-1 cells by pure DOTMA liposomes.** CV-1 cells were incubated (5 h, 37 °C) with liposomes associated with 100 μg of T7X556 and 1 μg of GTCO, or with 1 μg of GTCO alone. Shown is an immunoblot of 50 μg of extract protein (*lanes G* and *H*); for quantitation, *lanes A–F* show immunoblots of 20–100 ng of purified T7X556 protein.

We next tested whether T7X556 and the GTCO reporter gene could be delivered in separate steps rather than as a preformed complex. In one series of experiments, CV-1 cells were first treated with liposome-associated GTCO; after two different incubation periods (Fig. 4*A*, experiments I and II) the cells were washed and the exposed to liposome-associated T7X556; in parallel experiments (Fig. 4*A*, experiment III), T7X556 was delivered first, followed by GTCO. Enhancement by T7X556 was observed in all cases, although at a somewhat lower level when T7X556 was delivered first. Whereas we have not assessed directly the efficiencies of these protocols relative to that using the performed complex, it is apparent that co-delivery of T7X556 together with a receptor plasmid is not essential. Moreover, it appears that the order of addition of the regulatory protein and reporter gene is not a crucial determinant for detection of T7X556 enhancement activity and that the GRE binding activity of T7X556 is competent both *in vivo* and *in vitro*. The results also argue against the possibility that the elevated CAT activity observed in the presence of T7X556 is due simply to nuclear targeting of the reporter gene by the nuclear localization activity of T7X556 (28).

The order-of-addition experiments indicate that it should also be possible to assay T7X556 action on endogenous genes regulated by the receptor. However, co-delivery of the regu-

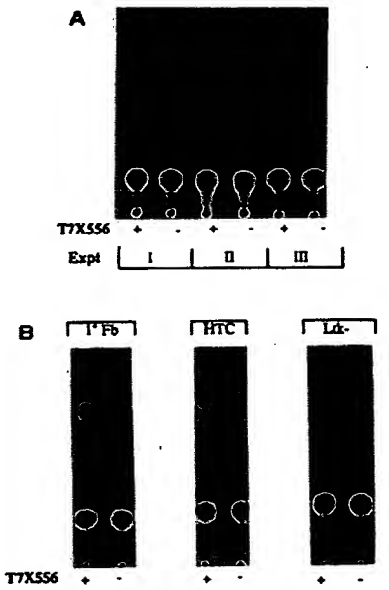

FIG. 4. **Separate delivery of T7X556 and reporter plasmids into CV-1 cells, and delivery of the T7X556-GTCO complex into various recipient cells.** *A*, order of delivery of 125 μg of T7X556 and 0.25 μg of GTCO into CV-1 cells. Pure DOTMA liposomes associated separately either with GTCO or with T7X556 were added to cells in different orders and with different time courses (+, separate addition of GTCO and T7X556; −, addition of GTCO only). In experiment I, cells were incubated with GTCO-liposomes for 3 h, washed for 1 h, and then incubated with T7X556-liposomes. In experiment II, cells were incubated with GTCO-liposomes for 3 h, with DME with FCS for 12 h, washed for 1 h, and then incubated with T7X556-liposomes. In experiment III, cells were incubated for 3 h with T7X556-liposomes, washed for 1 h, and then incubated with GTCO-liposomes. *B*, delivery into different recipient cells. In each case, 2.5 μg of GTCO and 125 μg of T7X556, associated with DOTMA liposomes as in Fig. 2, were delivered into primary rat embryo fibroblasts (*1 °Fb*), HTC cells, or Ltk$^-$ cells. All cultures were maintained in DME medium with FCS in 7% CO_2 at 37 °C.

latory protein with exogenous reporter genes has certain advantages. First, co-delivery likely results in the introduction of multiple reporter genes, thus increasing the sensitivity of the assay for regulatory protein activity. Second, the co-delivery strategy allows regulatory activity to be assayed on the same reporter gene in multiple cell types, without complications arising from chromsomal position effects. For example, we tested liposome-mediated co-delivery of T7X556 and GTCO into cells from three diverse sources: 6.10.2, a glucocorticoid receptor-deficient mutant (26) derived from HTC, an established line of rat hepatoma cells; Ltk^-, an established mouse cell fibroblast line that produces endogenous mouse glucocorticoid receptor; and primary cultures of rat embryo fibroblasts, which produce endogenous rat glucocorticoid receptor. In all three types of cell cultures, T7X556 strongly enhanced CAT expression from GTCO (Fig. 4*B*). Notably, the endogenous glucocorticoid receptor in the L cells and rat embryo fibroblasts did not interfere in these assays, as no hormone was added to the cultures. Thus, the constitutive activity of T7X556 can be demonstrated in primary cells as well as in established lines, including those containing endogenous glucocorticoid receptors, via liposome-based protein-DNA co-delivery.

In summary, we show that a fragment of the glucocorticoid receptor that has been overproduced in *E. coli* and purified to homogeneity retains the potential for transcriptional enhancement at GRE-linked promoters *in vivo*. In addition, we suggest that DOTMA liposome-mediated delivery may be a generally applicable method for introducing proteins in functional form into many cell types. In principle, for example, the activity of tissue- or developmental stage-specific transcription factors could be assessed in cell types that lack these factors. Other than microinjection, a technique that requires specialized equipment and the capacity to assay individual injected cells, few procedures have been described for introducing proteins into living cells (23, 24, 29, 30). Somewhat surprisingly, we were unsuccessful in adapting directly various DNA transfection procedures for protein delivery. The DOTMA liposome procedure, while rather inefficient, nevertheless yielded enhancement levels of up to 70-fold by the T7X556 receptor derivative. In principle, DOTMA liposome-based delivery should permit functional assessment of the *in vivo* activities even of proteins that lack established assays for measuring their activities *in vitro*. For example, only recently has enhancement activity been detected *in vitro* for T7X556 (31), and the activities observed in vitro are substantially lower than the apparent *in vivo* activity observed after liposome delivery. It is conceivable that covalent modifications of the protein (*e.g.* phosphorylation) or association with other intracellular proteins may occur *in vivo* but not *in vitro* and that our results imply a role for such modifications as well as a functional assay for their effects.

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